Journal of Medical Virology 57:217–222 (1999)

Antiviral Effects of Antisense RNA on Hepatitis C Virus RNA Translation and Expression

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We developed approaches using antisense RNA to inhibit hepatitis C virus (HCV) RNA translation and HCV core protein expression. An HCV genotype 1b cDNA comprising nt 1-1321 or a fusion construct consisting of HCV (nt 1-584) and luciferase cDNAs were inserted downstream of T7 and CMV promoter sequences and used to generate HCV RNA target molecules. Such constructs will produce HCV core or HCV core–luciferase fusion proteins in vitro or within transfected cells. Seven different antisense RNA constructs were designed to target the highly conserved 5′ region of HCV RNA at nt positions 1-402. For in vitro experiments, synthesized HCV RNA target sequences and antisense RNAs were mixed at various molar ratios and subsequently translated in a rabbit reticulocyte lysate system. In cell culture studies, the HCV core–luciferase fusion cDNA was co-transfected with antisense RNA-producing constructs into human hepatocellular carcinoma (HCC) cells. Luciferase activity in cell lysates was measured to determine quantitatively antiviral effects within the cell. It was found that translation of HCV RNAs was efficiently inhibited by antisense RNA in vitro. The specificity of this inhibition was confirmed using control target RNA sequences or nonrelevant antisense RNA constructs. Co-transfection studies demonstrated that antisense RNA inhibited HCV core–luciferase fusion protein expression by 41–57% in HuH-7 HCC cells. These studies indicate that antisense RNA will find viral target RNA sequences in HuH-7 cells and inhibit HCV RNA translation. More important, these studies have defined critical viral RNA target sequences susceptible to antisense inhibitory effects within the cell. ***J. Med. Virol. 57:217–222, 1999.***

KEY WORDS: antisense RNA; hepatitis C; core–luciferase fusion

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of post-transfusion hepatitis [Choo et al., 1989; Kuo et al., 1989]. The HCV carrier rate varies between 0.5–1.5% of the general population in many countries of the world [Esteban et al., 1990; Katayama et al., 1990; Kuo et al., 1989]. In the setting of persistent viral infection of the liver, individuals often progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [Kiyosawa et al., 1990; Saito et al., 1990]. Effective treatment of chronic HCV infection is important in prevention of complications of the liver disease. However, current therapy with interferon-α administration is only partially effective, since 15–25% of patients with chronic HCV infection show a favorable virologic response, and this response depends in part on the HCV genotype and the viral load [Yoshioka et al., 1992]. Thus, there is a need to develop additional antiviral approaches in an attempt to reduce the frequency of persistent viral infection and the long-term clinical consequences of this disease.

Nucleic acid-based therapy using intracellular immunization techniques has been employed as an antiviral approach for human immunodeficiency virus (HIV) infection [Baltimore, 1988]. This approach may also have value for HCV infection, as well as a means to inhibit viral gene expression. In this regard, antisense RNA and DNA have been actively studied as a means to inhibit gene expression and viral replication [Izant and Weintraub, 1984; Koschel et al., 1995; Milligan et al., 1993; Tonkinson and Stein, 1993]. Our previous investigations have demonstrated that antisense oligo-

This work was presented at the 1995 Annual Meeting of the American Association for the Study of Liver Diseases, and appeared in abstract form.

Grant sponsor: NIH; Grant numbers: CA-35711, AA-02169.

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Accepted 5 November 1997

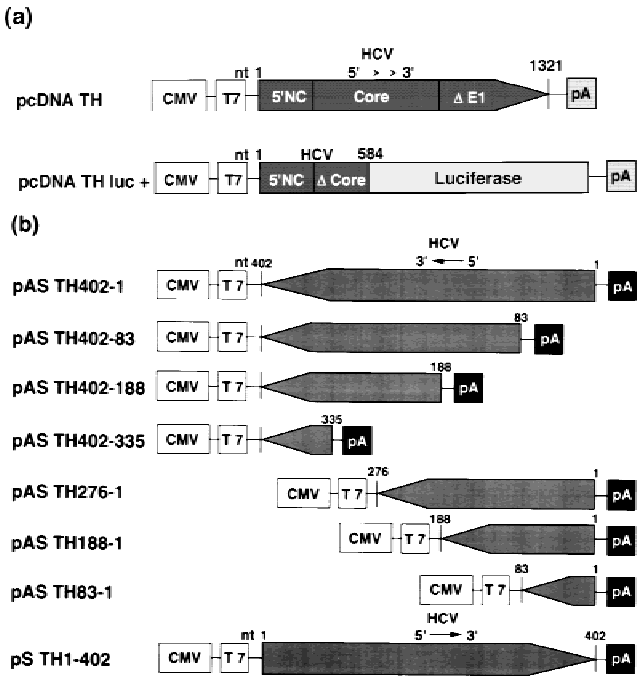

Fig. 1. Diagrams of HCV expression vectors and series of antisense constructs driven by CMV and T7 promoters. **a:** An HCV cDNA type 1b was inserted into pcDNA3 to make the pcDNATH expression vector (see text). The pcDNATHluc+ construct has an insert that produces a HCV (nt position 1-580) and luciferase fusion protein. **b:** Organization of the antisense HCV RNA constructs. The numbers following the TH in the construct's names indicate the nt positions of the HCV inserts.

deoxynucleotides (ODN) effectively inhibit HCV RNA translation in vitro, and these studies have partially defined critical viral target sequences susceptible to antisense effects [Wakita and Wands, 1994]. However, many difficulties exist regarding the use of short ODNs to inhibit the viral life cycle in infected cells [Stein and Cheng, 1993]. As another approach, we have tested—for the first time—antisense RNA constructs as inhibitors of HCV RNA translation, and found that antisense RNA could substantially reduce HCV core protein expression not only in vitro but also in intact cells. Such constructs are viable candidates to be placed into expression constructs such as adenovirus for study of antiviral effects in hepatocytes or intact mammalian liver [Scaglioni et al., 1996].

MATERIALS AND METHODS

Expression Vectors

The HCV (genotype 1b) cDNA TH was cloned from a patient serum [Wakita and Wands, 1994]. This TH cDNA contains the 5′ noncoding region, core and partial E1 region (nt 1-1321), was inserted downstream of CMV and T7 promoter sequences of the pcDNA3 vector (Invitrogen, San Diego, CA), and was subsequently named pcDNATH (Fig. 1a). Plasmid pcDNATHluc+ was prepared by fusing the firefly luciferase gene (with the translation initiation codon deleted) in frame with the first 584 nt of HCV. This construct expresses a fusion molecule consisting of the N-terminal 82 amino acids of the HCV core protein, as well as the entire luciferase protein under the control of CMV and T7 promoters.

Antisense Constructs

A series of antisense HCV RNA expression vectors were constructed as described in Figure 1b. To generate pASTH402-1, an HCV cDNA containing nt 402 to 1 was inserted downstream of CMV and T7 promoters to express an antisense RNA directed against the positive strand HCV target RNA. The other antisense constructs were derived from pASTH402-1 by 5′ and 3′ deletions of the insert. pASTH402-83, pASTH402-188, pASTH402-276, and pASTH402-335 contain antisense HCV inserts comprised of nt 402-83, 402-188, 402-276, and 402-335, respectively. pASTH276-1, pASTH188-1, and pASTH83-1 have antisense HCV sequences represented by nt 276-1, 188-1, and 83-1. The control construct, pSTH1-402, contains a sense HCV insert containing nt 1-402. The control vectors, pASGEMNae and pASGEMHind, contain 400 and 80 nt length inserts, respectively, of nonrelevant DNA derived from pGEM-11Zf(+) (Promega, Madison, WI). The luciferase plasmid (Promega) was used as a template to transcribe control luciferase RNA.

In Vitro Transcription and Translation

Transcripts were produced by T7 RNA polymerase (Promega, Madison, WI) from the linearized pcDNATH and pcDNATHluc+ vectors. Synthesized RNAs were extracted with phenol/chloroform and precipitated with ethanol. One picomole of synthesized RNA was heated at 70°C for 5 min and subsequently translated in a rabbit reticulocyte lysate (RRL) system (Promega, Madison, WI). After incubation at 30°C for 15 min, translated products were analyzed by SDS-PAGE and autoradiography was performed after translations were carried out in the presence of ^{35}S methionine (DuPont NEN, MA). The signal intensities of translated core protein were determined by densitometric scanning. For the HCV–luciferase fusion constructs, luciferase assays were performed as described [Ausubel et al., 1989] and relative light units (RLU) were measured using a LKB-Wallac 1251 Luminometer (Wallac, Turku, Finland).

In Vitro Antisense RNA Assay

Antisense RNAs were also transcribed in vitro from the above described antisense constructs (pAS) by T7 RNA polymerase. A 10-fold molar excess of synthetic antisense RNA and one picomole of target RNA was denatured separately and then mixed. These RNA mixtures were translated in RRL system. Translated products were assayed by SDS-PAGE or for luciferase activity as described above. Percent inhibition was calculated by comparison with control translations carried out in the absence of antisense RNA.

Transfection of HuH-7 Cells and Evaluation of Antisense RNA Effects

Fifty or 100 ng of pcDNATHluc+ plasmid DNA along with 2 or 4 μg of pAS constructs (1:40 ratio by weight) were transfected into HuH-7 cells by a modified calcium phosphate precipitation method. [Chen and Okayama, 1987]. In addition, 1 μg of pSVβgal vector (Promega, Madison, WI) was also co-transfected to normalize transfection efficiency. Luciferase activity was measured in cell lysates 10 or 24 hr after transfection. β-galactosidase activity in the transfected cell lysates was measured with the β-galactosidase enzyme assay system (Promega, Madison, WI). Luciferase activity derived from each transfection was also corrected for transfection efficiency by β-galactosidase activity. In some experiments, human growth hormone was also used to normalize transfection efficiency in HuH-7 cells. In this regard, the pXGH5 vector (Nichols Institute Diagnostics, San Juan Capistrano, CA) was co-transfected and growth hormone levels in the culture supernatants were measured using the HGH TGES Radio immunoassay system (Nichols Institute Diagnostics). Antisense RNA effects in HuH-7 cells were evaluated by luciferase activity as measured in the cell lysates following co-transfection with antisense constructs and control antisense RNA.

RESULTS

Specific Inhibition of HCV RNA Translation by Antisense RNA In Vitro

HCV RNA was translated in vitro as 34 kDA core–truncated envelope fusion protein (Fig. 2a). This fusion protein was identified with a monoclonal antibody prepared against the HCV core protein [Moradpour et al., 1997]. The 34 kDa fusion protein was subsequently processed to a 21 kDa core protein in the presence of microsomal membranes. This phenomenon has been demonstrated previously [Wakita and Wands, 1994]. In vitro experiments demonstrating dose-dependent inhibition of HCV RNA translation by antisense RNA, and the results are summarized in Figure 2. In these studies, antisense RNA transcribed from the pASTH402-1 vector was mixed at the indicated molar ratio with target HCV TH RNA (nt 1-1321). The degree of inhibition of HCV RNA translation increased from 32% to 87% with additions of higher levels of antisense RNA.

Effects of Shorter Antisense RNA Constructs In Vitro

To determine the most effective sequence for inhibition of HCV RNA translation, six antisense constructs with shorter inserts were derived from the parental pASTH402-1-containing vector. A 20-fold molar excess of antisense RNA was mixed with HCV or luciferase target RNA, and translated in the presence of ^{35}S methionine. Translated products were separated on SDS-PAGE followed by autoradiography. After densitometry scanning of the X-ray film, the percent inhibition

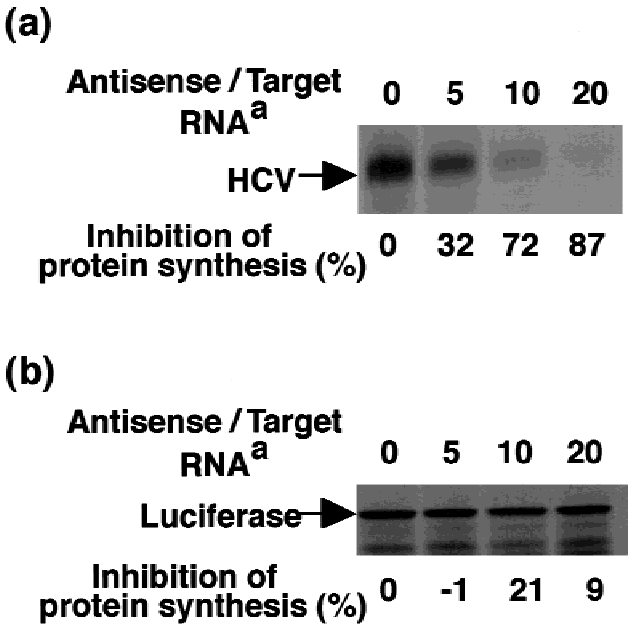

Fig. 2. Dose-dependent inhibition of HCV RNA translation by antisense RNA. Antisense RNA was synthesized from the pASTH402-1 vector in vitro and mixed with the target HCV RNA nt1-1302 **(a)** or control Luciferase RNA. **(b)** Antisense and target RNAs were mixed at the indicated molar ratio varying from 5:1 to 20:1. Inhibition of protein synthesis was evaluated by comparing the signal intensities of HCV or Luciferase protein synthesis by densitometric scanning. [a]Antisense RNA was mixed with 1 picomole of target RNA at the indicated molar ratios.

exhibited by each antisense RNA species was calculated by comparing the signal intensities of expressed proteins. The ASTH402-1, ASTH402-83, ASTH402-188, and ASTH402-335 RNAs were found to effectively inhibit HCV core protein translation (91.0, 79.4, 73.4, and 97.5%, respectively), while there was no effect of these antisense RNAs on the translation of a control luciferase RNA species, (–2.1, –8.4, –7.1, and 4.7%, respectively; Fig. 3a and b).

Antisense RNA synthesized from constructs with deletion in the 5′ region of pASTH402-1 were not effective in inhibiting HCV RNA translation as compared to antisense RNA species derived from the 3′ deleted constructs shown in Figure 4. There were no antisense effects on HCV protein synthesis exhibited by ASTH 276-1, 188-1, 83-1 RNAs compared to the "full-length" ASTH 402-1 (–9.8, –11.1 and –8.4%, respectively; Fig. 4). Finally, nonrelevant ASGEMHind and ASGEMNae RNAs were transcribed from the control constructs. RNAs synthesized from both of these constructs had no inhibitory effects on HCV RNA translation in vitro (–7.8 and –1.9%; Fig. 4).

Antisense RNA Effect on Translation of a HCV-Luciferase Fusion RNA

A HCV-luciferase fusion target RNA molecule was synthesized from a linearized pcDNATHluc+ plasmid and subsequently translated in the RRL system. HCV core– and luciferase–fusion proteins appeared as a 70

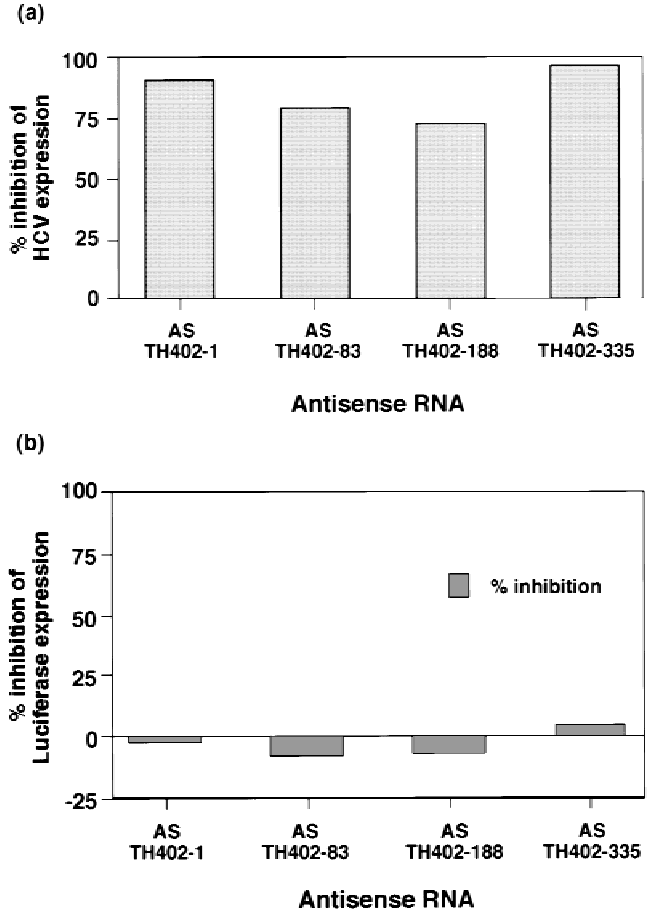

Fig. 3. Percent inhibition of HCV and Luciferase RNA translation by antisense RNA in vitro. A 20:1 molar excess of antisense RNA was mixed with target HCV **(a)** or luciferase **(b)** RNA. The ASTH402-1, 402-83, 402-188, and 402-335 antisense RNAs were synthesized from pASTH402-1, 402-83, 402-188, and 402-335, respectively. The percent inhibition of HCV translation exhibited by each antisense RNA is depicted.

kDa species (where 8 kDa represents the HCV core and 62 kDa represents the luciferase moiety of the fusion protein) on SDS-PAGE gels (data not shown). Two microliters of each translation mixture were assayed for luciferase enzymatic activity and quantitated by RLU. THluc+ RNA translations were inhibited by antisense RNAs. The most potent effect (56%) was derived from the ASTH402-1 construct (Table 1). The specificity of the inhibitory effect of ASTH402-1 was confirmed by the lack of inhibition using a control antisense RNA target sequence (ASGEMNae).

Antisense RNA Effects on HCV Gene Expression in HCC Cells

Antisense effects of pASTH constructs were evaluated following co-transfection experiments in HuH-7 cells. The pcDNATHluc+ expression vector was co-transfected along with the antisense RNA constructs; the pSVβgal or pXGH5 reporter plasmids were employed to normalize transfection efficiency. HCV–core–luciferase fusion protein expression was measured by luciferase activity in cell lysates, and used as a "read

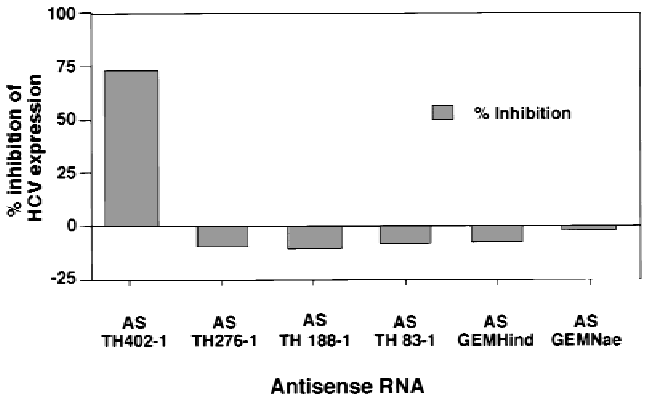

Fig. 4. Effects of antisense RNAs on HCV translation as synthesized from vectors with 5′ deletions of the parental pASTH402 construct. Such deletions abolished antisense RNA effects. ASGEMHind and ASGEMNae are control antisense RNAs derived from plasmid DNA sequences. Percent inhibition of each antisense RNA is indicated by the bars. Minus inhibition represents the enhancement of translation of HCV RNA by antisense RNA.

TABLE I. Antisense RNA Effects on HCV–Luciferase Hybrid RNA Translation as Measured by Luciferase Activity In Vitro

Template/antisense RNA	RLU[a]	Percent inhibition (%)
THluc+/(–)	57,202	0
THluc+/ASTH 402-1	24,977	56
THluc+/ASTH 402-83	32,697	43
THluc+/ASTH 402-188	29,182	49
THluc+/ASTH 402-335	43,957	23
THluc+/ASTH 276-1	62,567	–9
THluc+/ASGEMNae	53,137	7

[a]RLU, relative light unit.

out" for intracellular antisense RNA effects. The results of these investigations are summarized in Table 2. In experiments (a) and (b), β-galactosidase activity was assayed, and luciferase activity was subsequently corrected according to the transfection efficiency, as shown in the "corected RLU" column. In experiment (c), the amount of growth hormone in culture supernatant was determined and luciferase activity corrected for transfection efficiency. Under these experimental conditions, the pASTH402-1 construct showed 41–57% inhibition of HCV core–luciferase fusion protein expression within the cell.

DISCUSSION

Because the therapeutic effects of interferon-α administration are limited in chronic HCV infection, several other antiviral compounds such as ribavirin have been previously evaluated [Kakumu et al., 1993]. However, the action of these agents on HCV replication appear to be limited and transient. Previously, it was demonstrated that certain antisense ODN effectively and specifically inhibited HCV RNA translation in vitro, and these studies partially defined critical viral RNA target sequences accessible to antisense ODN effects [Wakita and Wands, 1994]. Moreover, others have reported that antisense phosphodiester and phosphorothioate ODNs will inhibit HCV translation as well

TABLE II. Antisense RNA Inhibition of HCV Expression by pcDNA TH luc+ in HuH7 Cells

Co-transfected vector	RLU[a]	β-galactosidase activity (A405)	Corrected RLU[b]	Percent inhibition of luciferase activity (%)
10-hr culture/β-gal				
pASTH 402-1	3,205	0.182	3,592[c]	41
pASTH 402-83	7,021	0.356	4,023	34
pASTH 402-188	7,360	0.333	4,550	26
pASTH 402-335	7,765	0.240	6,600	−8
pSTH 1-402	7,662	0.247	6,328	−3
pASGEMNae	6,131	0.204	6,131	0
24-hr culture/β-gal				
pASTH 402-1	11,222	0.559	10,058[d]	57
pASTH 402-83	29,367	1.066	13,802	42
pASTH 402-188	35,702	1.064	16,811	29
pASTH 402-335	46,517	1.186	19,650	17
pSTH 1-402	47,087	1.190	19,142	16
pcDNA 3	23,517	0.510	23,587	0
24-hr culture/GH				
pASTH 402-1	13,887	76,496	17,023	56
pSTH 1-402	35,627	96,520	34,613	10
pcDNA 3	38,490	93,772	38,490	0

[a]Mean values of duplicates.
[b]RLU was corrected by transfection efficiency to pcDNA3.
[c]RLU was corrected by transfection efficiency to pASGEMNae.
[d]Mean values of triplicates.

[Alt et al., 1995; Hanecak et al., 1996; Mizutani et al., 1995; Seki and Honda, 1995; Vidalin et al., 1996]. However, efficient delivery of antisense ODN constructs to target cells or tissue types has been difficult to achieve [Stein and Cheng, 1993]. One major incentive for testing antisense RNA effects on HCV RNA translation within cells as compared to short (15–25 nt) ODNs was that antisense constructs may be delivered to hepatocytes by viral expression vectors—which are far more efficient vehicles for transfer of nucleic acids [Chatterjee et al., 1992].

In this study, it was first necessary to show that synthetic antisense RNA will specifically inhibit HCV RNA translation in vitro, and then to define the critical target sequences involved. Indeed, we found sequence-specific inhibition of HCV RNA translation using an antisense RNA molecule as short as 67 residues. The use of a pre-annealing step of target to antisense RNA before in vitro translation caused nonspecific translation inhibition of the control luciferase target RNA. However, once this pre-annealing step was omitted, specific inhibition by antisense RNAs of HCV target RNA molecules was observed. To determine the optimal antisense RNA sequence for this antiviral effect, we produced smaller constructs by 5′ and 3′ deletions of the parental pASTH402-1 and tested them for inhibitory effects. The 3′ deleted antisense constructs pASTH402-83, 402-188, and 402-335 were found to be effective, whereas the 5′ deleted constructs represented by pASTH276-1, 188-1, and 83-1 had no measurable antisense effects. Our previous experiments found antisense effects with ODN molecules targeting the region around and including the HCV core translation initiation codon. The present findings derived from antisense RNA species are in general agreement with these results, since the maximal antisense RNA effects were achieved using an RNA molecule targeted to the HCV core start codon and a highly conserved downstream sequence. Such studies emphasize the importance of this viral region as a target area for nucleic acid-based antiviral approaches.

To establish a system to evaluate antisense RNA effects within intact cells, the pcDNATHluc+ expression vector was developed. HCV core and firefly luciferase proteins were expressed as a fusion molecule both in vitro and in HCC cells following transient transfection. This model system provided a sensitive and quantitative approach to evaluate antisense RNA effects, since only small amounts of the expression vector were needed to produce low levels of target RNA within HuH-7 cells. There were significant differences in the results when using pcDNATH and pc-DNATHluc+ with respect to antisense effects on translation of HCV RNA. For example, only the antisense RNA generated from the pASTH402-1 construct was effective in vitro against the target THluc+ RNA species. This finding was also observed in transfected HuH-7 cells.

It is noteworthy that the viral region targeted by pASTH402-1 is highly conserved among all HCV genotypes. This domain appears not only to possess a stable secondary structure [Brown et al., 1992], but also has a putative IRES function believed to be essential for initiation of translation [Tsukiyama-Kohara et al., 1992] in cooperation with a sequence downstream of the core AUG codon [Reynolds et al., 1995]. Viral escape mutants are known to occur during natural infection. However, it seems unlikely that viral mutations will lead to escape from pASTH402-1 antisense RNA effects due to the important biologic functions subscribed to this region during the life cycle of the virus and the size of the antisense RNA molecule. The intracellular mechanisms of antisense RNA effects may depend on

duplex formation with target RNA. Formation of duplex RNA may interfere with the function of the viral RNA or promote RNA degradation by disruption of stable secondary structure.

Future studies will need to focus on antisense RNA molecules as delivered by retroviral or adenoviral vectors in cells stable-transfected with subgenomic regions of HCV [Scaglioni et al., 1996]. Primary culture of chimpanzee hepatocytes which support viral replication of HCV to a limited extent may be of interest in this regard [Lanford et al., 1994]. We have recently developed inducible cell lines that express the HCV core RNA and protein under the control of a tetracycline-sensitive promoter [Moradpour et al., 1996]. This system will be used to determine the efficiency of antisense RNA molecules under conditions of varying amounts of target viral RNA, since the expression of HCV RNA and protein is tightly regulated by adjusting the concentration of tetracycline in the culture medium. Finally, efficient cell-specific delivery methods of antisense RNA will need to be developed and employed to test for possible antiviral effects in vivo. Recombinant adenovirus-mediated gene transfer has recently been shown to be a promising cell delivery system in this regard [Zabner et al., 1993] and has recently been used to inhibit HBV replication in HEPG2215 cells [Scaglioni et al., 1996]. As shown in the present investigation, transient expression of antisense RNA in cells may suppress the level of HCV RNA translation and enhance the efficiency antiviral effects.

ACKNOWLEDGMENTS

We thank Miss Atsuko Fujisawa for preparation of this manuscript. Dr. Darius Moradpour was supported by fellowships from the Swiss National Science Foundation and the Roche Research Foundation.

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